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Microbial conversion of *p*-hydroxyphenylacetic acid to homogentisic acid[1,2]

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An unidentified bacterium degrades *p*-hydroxyphenylacetic acid by a pathway which involves homogentisic acid as an intermediate. Extracts of cells grown on *p*-hydroxyphenylacetic acid contain an enzyme that converts *p*-hydroxyphenylacetic acid to homogentisic acid. The enzyme has been partially purified by ammonium sulfate fractionation and some of its properties examined. The complete oxidation of 1 mole of *p*-hydroxyphenylacetic acid requires 1 mole of either NADH or NADPH, and 1 mole of oxygen, and results in the production of 1 mole of homogentisic acid. The enzyme preparation has a high specificity for *p*-hydroxyphenylacetic acid, but has about 25% activity with *p*-hydroxyphenylpyruvic acid.

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Une bactérie non-identifiée décompose l'acide *p*-hydroxyphénylacétique par un chemin qui implique l'acide homogentisique comme un intermédiaire. Des extraits de cellules qui se sont développées sur l'acide *p*-hydroxyphénylacétique contiennent un enzyme qui convertit l'acide *p*-hydroxyphénylacétique en acide homogentisique. L'enzyme a été partiellement purifié par fractionnation avec le sulfate d'ammonium et quelques-unes de ses propriétés ont été examinées. L'oxidation complète de 1 mole d'acide *p*-hydroxyphénylacétique exige 1 mole soit de NADH ou de NADPH, et 1 mole d'oxygène, et résulte en la production de 1 mole d'acide homogentisique. La préparation enzymatique a une haute spécificité pour l'acide *p*-hydroxyphénylacétique, mais environ 25% d'activité avec l'acide *p*-hydroxyphénylpyruvique.

Introduction

Aerobic microbial degradation of phenolic compounds usually involves the introduction of a second hydroxyl group ortho or para to the first hydroxyl group before oxidative cleavage of the ring (6, 7, 9, 11–15, 23, 24). Accordingly, the microbial degradation of *p*-hydroxyphenylacetic acid (*p*HPA) would be expected to proceed by a pathway that involved 3,4-dihydroxyphenylacetic acid (DHPA) as an intermediate and, in fact, the conversion of *p*HPA to DHPA has been shown to occur in several strains of *Pseudomonas* (1, 3, 8, 20).

During studies on the degradation of aromatic compounds by an unidentified bacterium isolated from soil, it was observed that the organism after adaption to growth on *p*HPA was unable to use DHPA, or other mono- and di-hydroxyphenylacetic acids. However, extracts of the cells contained high levels of homogentisic acid oxidase, which was not present in extracts of cells grown on non-aromatic carbon sources. These results suggested that homogentisic acid was an intermediate in the degradation of *p*HPA. This paper describes an enzyme-catalyzed oxidation of *p*HPA to form homogentisic acid (Fig. 1), and suggests that the reaction involves migration of the side chain.*

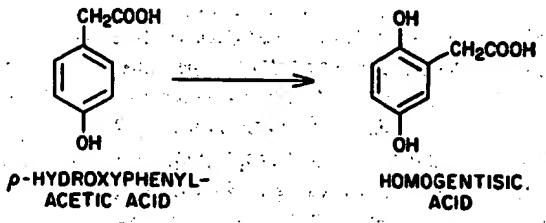

FIG. 1. The conversion of *p*-hydroxyphenylacetic acid to homogentisic acid.

Materials and Methods

Isolation and Description of the Organism

The organism, PRL W19, was isolated from garden soil by enrichment culture using 4-phenylbutyric acid as a sole carbon source, and grown through several transfers on a medium containing 0.2% 4-phenylbutyric acid, 0.1% yeast extract, 0.15% KH_2PO_4, 0.35% K_2HPO_4,

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*For reasons outlined in Discussion and to differentiate from enzymes catalyzing the conversion of *p*HPA to DHPA, the enzyme in this paper has been called *p*-hydroxyphenylacetate-1-hydroxylase.

0.1% NH_4NO_3, 0.05% $MgSO_4 \cdot 7H_2O$, and 0.001% f each of the f llowing: $ZnSO_4 \cdot 7H_2O$, $NaMoO_4 \cdot 7H_2O$, $FeSO_4 \cdot 7H_2O$, and $CaCl_2 \cdot 2H_2O$. The culture was streaked on the above medium solidified with agar, and a single colony isolated for further study. The organism was maintained by transfer to fresh medium twice a week.

The organism is a gram-negative, ellipsoidal rod, motile with peritrichous flagella, growing singly or in chains of two to eight cells. The cells are pink when harvested from broth. Agar colonies are circular, entire to undulate, cream colored, and viscous. It grows in nutrient broth, producing a ring at the surface and a viscous sediment. It is an aerobic organism, growing only at the surface of Hugh and Leifson medium (17), producing acid. Acid is slowly produced from glucose, fructose, galactose, sucrose, maltose, lactose, D- and L-arabinose, D-xylose, and L-fucose, but not from D-lyxose, D-mannose, or D-allose. On Hutner's basal medium (18), the following compounds are used: glycine, benzylamine, hippurate, acetate, butyrate, caproate, succinate, tartrate, malate, lactate, pyruvate, benzoate, *o*-hydroxybenzoate, phenylacetate. Citrate, meso-inositol, D-arabitol, adonitol, dulcitol, sorbitol, and trehalose did not support growth. Growth occurs only at the surface of nutrient gelatin, with no liquifaction. Litmus milk becomes alkaline at the surface, with no coagulation. Indole is not produced, the Voges-Proskauer test is negative, nitrates are used but nitrites do not accumulate. Starch is not hydrolyzed and H_2S is not produced in lead acetate agar. The organism may be a species of *Alcaligenes* or *Achromobacter*, but has not yet been positively identified.

Growth of the Organism

For the preliminary studies the organism was grown in 100 ml of salts medium described above, containing 0.2% *p*HPA in 500-ml Erlenmeyer flasks on a rotary shaker. When larger amounts of cells were required for the preparation of cell extracts, the cells were grown for 18 h by forced aeration in 3 liters of medium contained in 5-liter bottles or in 20 liters of medium in stirred fermentors. The medium initially containing 0.3% potassium gluconate was then supplemented with 0.05% *p*HPA and grown for a further period of 5 h. The cells were harvested and washed with water by centrifugation.

Preparation and Treatment of Cell Extracts

The cells were suspended in two volumes of 0.05 *M* bicine (N,N-bis(2-hydroxyethyl)glycine) buffer, pH 7.5; cooled to 5°C; and broken by three treatments of 20 s with a Branson probe sonicator, with cooling to 5°C between each treatment. The unbroken cells and cell debris were separated by centrifugation at 20 000 × *g* for 20 min. The crude extract was mixed with 0.1–0.25 volumes of 2% protamine sulfate and centrifuged. Since excess protamine sulfate resulted in losses of enzyme activity, the amount of protamine sulfate to be added had to be determined for each batch of cells. The supernatant from the protamine sulfate treatment was mixed with 0.75 volumes of a saturated ammonium sulfate solution (43% AMS) and centrifuged. Solid ammonium sulfate was then added t the supernatant to 65% saturation (65% AMS) and centrifuged. The protein precipitates were dissolved in a volume f buffer (0.05 *M* bicine, pH 7.5; 0.01 *M* dithiothreitol) equal to one-third of the original volume f initial crude extract.

Assays of Activity

The consumption of oxygen by intact cells and cell extracts was determined in a Warburg apparatus at 30°C. For the experiments with intact cells, each flask contained 1.0 ml 0.1 *M* phosphate buffer, pH 7.0; 20 mg cells (wet weight) suspended in water; 5 μmoles substrate added from the side arm; and water to a final volume of 3.0 ml. The center well contained 0.2 ml 20% KOH to absorb carbon dioxide. The production of carbon dioxide was measured by the Direct Method (28) at pH 4.8. For these experiments the cells were suspended in 1.0 ml tris (tris(hydroxymethyl)aminomethane), water was added, and the pH adjusted to 4.8 with dilute phosphoric acid. The mixture was placed in the main compartment of the Warburg flask and the substrate added from the side arm.

p-Hydroxyphenylacetate hydroxylase was determined by measuring the rate of decrease in optical density at 340 nm resulting from the disappearance of reduced nicotinamide adenine dinucleotide (NADH). The reaction mixture contained 1.0 ml of 0.1 *M* bicine buffer, pH 7.5; 0.5 μmoles NADH; 0.025 μmoles flavine adenine dinucleotide (FAD); 5 μmoles *p*HPA; 1 μmole reduced glutathione; 10 μmoles $MgSO_4$; and enzyme and water to 3.0 ml. Other additions were made as indicated in the text. One unit of activity is that amount of enzyme which catalyzed the disappearance of 1 μmole NADH per minute, using 6.22×10^6 cm^2/mole as the extinction coefficient of NADH. NADH was not suitable for measuring the consumption of oxygen in the presence of *p*HPA, because of the high values of oxygen consumption obtained in the absence of *p*HPA. For studies involving the consumption of oxygen, reduced nicotinamide adenine dinucleotide phosphate (NADPH) generated from glucose-6-phosphate dehydrogenase (Sigma, type XII) was used as the coenzyme.

Homogentisate oxygenase (EC. 1.13.1.5) was determined by measuring the increase in optical density at 330 nm resulting from the production of maleylacetoacetate from homogentisic acid in the absence of reduced glutathione. The reaction mixture contained 1.0 ml bicine buffer, pH 7.5; 0.3 μmole homogentisic acid; 1.0 μmole $FeSO_4$; and water to a volume of 3.0 ml. One unit of homogentisate oxygenase activity is defined as that amount of enzyme which catalyzes the change of one optical density unit per minute measured at 330 nm. The same reaction conditions were used for measuring the consumption of oxygen in the Warburg apparatus.

Isolation and Identification of Homogentisic Acid

The product of the reaction with *p*HPA was isolated from the reaction mixture, after acidification with HCl to pH 1, by continuous extraction with ether, or hand extraction with ethyl acetate. The extracts were dried with anhydrous $MgSO_4$ and concentrated, and the product identified by gas–liquid chromatography (g.l.c.), thin-layer chromatography, and nuclear magnetic resonance (n.m.r.) spectroscopy. For the g.l.c. experiments, the product was converted to the silyl derivate (2) using BSA (N,O-bis-(trimethylsilyl)acetamide) and chromatographed on columns of 10% SE 52 on Chromosorb W and 10% SE 30 on Chromosorb W. Thin-layer chromatography was

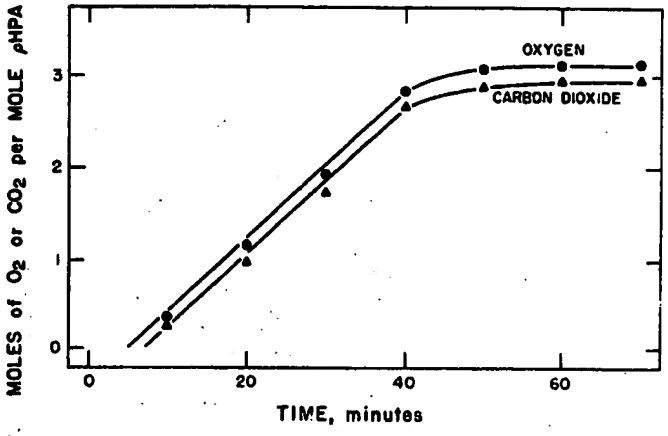

FIG. 2. The consumption of oxygen and production of carbon dioxide at pH 4.8 by *p*HPA-grown cells in the presence of 5 μmoles *p*HPA in the Warburg apparatus.

carried out on silica-coated sheets (Eastman Kodak 6060) using as solvents benzene, methanol, acetic acid: 45,8,4 (v/v); the upper layer of *n*-butanol, pyridine, water: 4,1,5 (v/v); and petroleum ether (b.p. 30–60°), ether, formic acid: 125,75,6 (v/v). Nuclear magnetic resonance (n.m.r.) spectra in dimethylsulfoxide-d_6 were obtained with a Varian Associates HA 100 spectrometer using tetramethylsilane as internal standard.

Analytical Methods

The production of homogentisic acid from *p*HPA during enzymatic reactions could be demonstrated by the colorimetric method of Stoner and Blivaiss (26), but the method was unreliable because of interference from components in the reaction mixture. Homogentisic acid in ethylacetate extracts was most satisfactorily determined after reaction with nitrous acid. For this procedure, samples of homogentisic acid (0.15 μmoles) dissolved in 2.8 ml water were mixed with 0.2 ml 1:1 mixture of 1% $NaNO_2$ and 1 *N* HCl and the optical density at 248 nm read after 3 min. *p*-Hydroxyphenylacetic acid was determined colorimetrically after reaction with diazotized *p*-nitroaniline (4) or by a modification of the method by Millons (25). Protein was determined by the method of Lowry *et al.* (22) using bovine serum albumin as a standard.

Results

Experiments with Intact Cells

Cells grown on *p*HPA consumed 3 moles of oxygen per mole of *p*HPA in the Warburg apparatus at pH 7.0. Such cells were not adapted to the use of *o*-hydroxyphenylacetic acid, *m*-hydroxyphenylacetic acid, 3,4-dihydroxyphenylacetic acid, homogentisic acid, DL-*p*-hydroxymandelic acid, or any of the corresponding derivatives of benzoic acid. *p*-Hydroxyphenylpyruvate was oxidized under similar conditions at about 25% of the rate with *p*HPA. However, total oxygen consumption exceeded that with *p*HPA. When the experiments were done at pH 4.8, *p*HPA was oxidized with the consumption of 3 moles of oxygen and the production of 3 moles of carbon dioxide per mole of substrate (Fig. 2). An explanation for the consumption of only 3 moles of oxygen per mole of *p*HPA was obtained by incubating washed aqueous suspensions of *p*HPA-grown cells with *p*HPA (pH 7.0) for periods calculated for optimum consumption of oxygen. The aqueous medium was acidified and extracted with ether. Examination of the ether extracts by thin-layer and gas–liquid chromatography showed the presence of

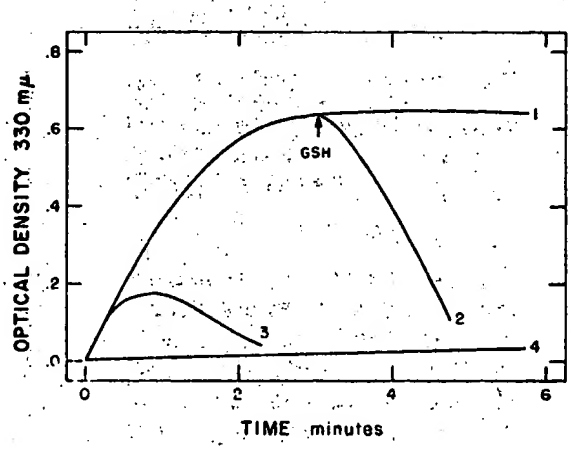

FIG. 3. The oxidation of homogentisic acid by extracts of *p*HPA-grown cells, measured at 330 mμ. 1. Conditions as described in Materials and Methods. 2. Reduced glutathione added at time indicated. 3. Reduced glutathione added at zero time. 4. Same as 1, except homogentisic acid was omitted.

TABLE 1

Fractionation of extracts f cells gr wn on *p*-hydroxyphenylacetic acid

	Vol., ml	Protein, mg	*p*-Hydroxyphenylacetate hydroxylase			Homogentisate oxygenase units	Ratio of activities*
			Units	% recovery	Specific activity		
Crude extract	34	743	90	100	0.12	105	0.86
Protamine sulfate supernatant	35	590	83	92	0.14	105	0.79
43% AMS	11.3	176	70	78	0.40	22	3.18
65% AMS	11.3	296	10	11	0.03	68	0.15

*Ratio = *p*-hydroxyphenylacetate hydroxylase: homogentisate oxygenase.

a compound corresponding in properties to *p*-hydroxybenzoic acid. The same compound was present in the medium when cells were grown on *p*-hydroxyphenylacetic acid. Since this organism was found to be unable to metabolize *p*-hydroxybenzoic acid, the consumption of only 3 moles of oxygen per mole of *p*HPA appears to be due to a side conversion of *p*HPA to *p*-hydroxybenzoic acid.

Experiments with Crude Extracts

Crude extracts of *p*HPA-grown cells supplemented with 2×10^{-3} *M* ferrous ion catalyzed the consumption of about 1 mole of oxygen per mole of homogentisic acid and converted homogentisic acid to a compound absorbing at 330 nm, which disappeared upon the addition of 1 μmole reduced glutathione (Fig. 3). These are the expected results for the conversion of homogentisic acid to maleylacetoacetate, followed by isomerization to fumarylacetoacetate upon the addition of reduced glutathione (10). The homogentisate oxygenase from cells grown on *p*HPA appeared to have properties similar to that from cells grown in *o*-hydroxyphenylacetic acid or *m*-hydroxyphenylacetic acid. Cells grown on gluconate, succinate, or benzoate contain very low levels of homogentisic acid oxidase.

*p*HPA increased both the rate and total consumption of oxygen when crude extracts were supplemented with excess NADH or with nicotinamide adenine dinucleotide (NAD), ethanol, alcohol dehydrogenase, Fe^{2+} and Mg^{2+}, but the results were inconclusive because of the high values obtained in the absence of *p*HPA. Crude extracts also catalyzed a decrease in absorpti n at 340 nm as a result of NADH disappearance in the presence of *p*HPA.

Enzyme Fractionation

Treatment of crude extracts with protamine sulfate reduced the levels of "NADH oxidase." Nearly all of the *p*-hydroxyphenylacetate-1-hydroxylase activity was precipitated by ammonium sulfate at 43% saturation (Table 1). Homogentisate oxygenase activity was present in both the 43% AMS and 65% AMS fractions, but largely concentrated in the 65% AMS fraction. The addition of ferrous ion was required to obtain maximum homogentisate oxygenase activity and frequently the ammonium sulfate fractions were inactive toward homogentisic acid in the absence of added ferrous ion.

The protein in the 43% AMS could be dialyzed against 0.01 *M* bicine buffer, pH 7.5, for 4 h or chromatographed on columns of Sephadex G 100 or Biogel P10 without appreciable change in enzymatic properties or specific activity. However, attempts to fractionate the protein on a column of DEAE-Sephadex A 50 resulted in large losses in enzyme activity, although protein recovery was good.

Properties of p-Hydroxyphenylacetate Hydroxylase

Crude and partially purified extracts of cells adapted for growth on *p*HPA required reduced nicotinamide coenzymes for activity (Table 2). NADPH had only about 70% of the activity obtained with NADH. The addition of the supernatant from an active preparation which had been heated in a boiling water bath for 10 min, then centrifuged, increased the rate of reaction. Of a number of cofactors tested, only FAD satisfactorily substituted for the heated supernatant. The stimulating effect of FAD on

the reaction rate varied with different preparations, but it was not found possible to obtain a preparation dependent upon the additi n of FAD for activity.

The addition of Mg^{2+} to the reaction mixture stimulated the rate of reaction. The addition of other metallic cations such as Mn^{2+}, Co^{2+}, Ca^{2+}, Mo^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+} was without effect, or slightly inhibitory on the reaction rate. Maximum rate was obtained at 3.3×10^{-3} *M* Mg^{2+}, but a higher concentration (1×10^{-2} *M*) was slightly inhibitory (Table 2). The addition of 3×10^{-3} *M* α,α-dipyridyl or *o*-phenanthroline produced a 30% inhibition in reaction rate; higher concentrations of the inhibitors were without increased effect. Diethyldithiocarbamate and 8-hydroxyquinoline were less inhibitory. EDTA produced a maximum inhibition of 30% at 3.3×10^{-5} *M* concentration, which was reversed by the addition of Mg^{2+} or other cations. The results of these studies do not reveal the role of metallic cofactors in the enzyme reaction.

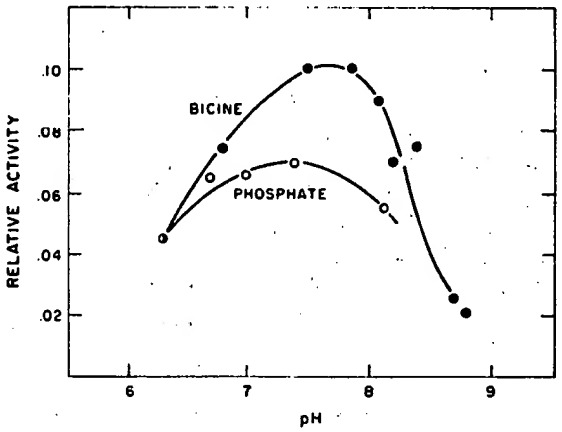

FIG. 4. The effect of pH on activity of *p*-hydroxyphenylacetate-1-hydroxylase.

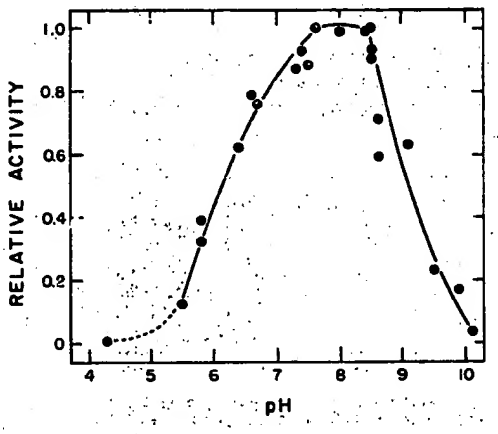

FIG. 5. The effect of pH on stability of *p*-hydroxyphenylacetate-1-hydroxylase.

TABLE 2

Factors affecting *p*-hydroxyphenylacetate-1-hydroxylase activity

Reaction conditions	Reaction rate, %
Complete reaction mixture	100
minus NADH	0
minus NADH, plus NADPH	69
minus FAD	76
minus Mg^{2+}	67
1.7×10^{-3} *M* Mg^{2+}	88
1×10^{-2} *M* Mg	88

NOTE: The complete reaction mixture contained 1.0 ml of 0.1 *M* bicine pH 7.5, 0.5 μmoles NADH, 0.025 μmoles FAD, 5 μmoles *p*HPA, 1 μmole reduced glutathione, 10 μmoles $MgSO_4$ (3.3×10^{-3}) and 0.1 unit of enzyme (about 0.16 mg protein) and water to 3.0 ml.

TABLE 3

Effect of inhibitors on *p*-hydroxyphenylacetate-1-hydroxylase

Inhibitor	Concn., *M*	% inhibition
Iodoacetate	3.3×10^{-2}	32
Iodoacetamide	3.3×10^{-3}	25
p-Chloromercuribenzoate	3.3×10^{-3}	97
	3.3×10^{-4}	30
Iodosobenzoate	3.3×10^{-3}	68
	3.3×10^{-4}	14
N-Ethyl maleimide	3.3×10^{-3}	95
	3.3×10^{-4}	77
Mercuric acetate	3.3×10^{-4}	98
	3.3×10^{-5}	41

TABLE 4

Substrate specificity of *p*-hydroxyphenylacetate-1-hydroxylase

Substrate	Relative rate
p-Hydroxyphenylacetate	100
p-Hydroxyphenylpyruvate	25
3,4-Dihydroxyphenylacetate	16
p-Hydroxyphenylpropionate	12
p-Hydroxybenzoate	3

NOTE: The following compounds were inactive under the conditions tested: phenylacetate, *o*-,*m*-hydroxyphenylacetate, *p*-hydroxybenzyl alcohol, phenylpyruvate, homogentisate, DL-tyrosine, 3,4-dihydroxyphenylalanine, *p*-methoxyphenylacetate, *o*-,*m*-,*p*-fluorophenylacetate, *o*-,*m*-,*p*-chlorophenylacetate

TABLE 5

Thin-layer chromatography of the product of reaction with *p*HPA

Solvent	R_f values reaction mixture	Homogentisic acid	*p*-Hydroxy-phenylacetic
Benzene, methanol, acetic acid	0.39, 0.91	0.39	0.91
Butanol, ether, water	0.44, 0.54	0.43	0.53
Petroleum ether, ether formic acid	0.13, 0.46	0.14	0.47

The enzyme reaction is strongly inhibited by several sulfhydryl inhibitors (Table 3) and indicates the essential role of —SH groups for enzyme action. Cleland's reagent (dithiothreitol) is more effective than reduced glutathione for the maintenance of activity in samples of stored enzyme. Samples of enzyme stored in the presence of Cleland's reagent maintain activity at −20°C for about 2 weeks with only about 10% loss in activity, while in the absence of Cleland's reagent, all activity is lost in about 3 days. The following compounds at 1×10^{-2} *M* concentration had no effect on the rate of reaction: semicarbazide, hydroxylamine, potassium fluoride, sodium arsenate, sodium arsenite, potassium cyanide, sodium azide, sodium borate.

The enzyme reaction was most active in bicine, MES (2(N-morpholino)ethane sulfonic acid) or tris-HCl buffers at pH 7.5–7.8, but had lower activity in phosphate buffer (Fig. 4). When stored at room temperature for 2 h, the enzyme was found to be most stable between pH 6.5 and 8.2 (Fig. 5).

p-Hydroxyphenylacetate-1-hydroxylase has a high specificity for *p*HPA. Acidic compounds having a free *p*-hydroxy group show low activity; the most active of these compounds is *p*-hydroxyphenylpyruvic acid (Table 4). When tested at equimolar concentrations, none of the compounds in Table 4 inhibited the oxidation of NADH in the presence of *p*-hydroxyphenylacetic acid. These results suggest that the enzyme has a high requirement for a substrate with a *p*-hydroxy group and an acetic acid side chain attached to an aromatic nucleus.

Identification of the Product of Reaction with pHPA

Ether or ethylacetate extracts of reaction mixtures involving the action of 43% AMS on *p*HPA contained a compound identical in properties with homogentisic acid when examined by gas–liquid and thin-layer chromatography. The results of thin-layer chromatography are shown in Table 5. The identity of the reaction product as homogentisic acid was confirmed by the n.m.r. spectrum. A signal was observed at 3.4 ppm representing the two methylene protons of the side chain, and eight signals were observed in the region 6.39–6.65 ppm representing the three aromatic protons with $J_{H\text{-}3,\,H\text{-}4} = 8$ Hz, $J_{H\text{-}4,\,H\text{-}6} = 3$ Hz, and $J_{H\text{-}3,\,H\text{-}6} = 1$ Hz. An identical spectrum was obtained of authentic homogentisic acid which was distinctly different from the spectrum of *p*HPA.

Stoichiometric Studies

Attempts to relate the consumption of oxygen with the oxidation of *p*HPA in the presence of

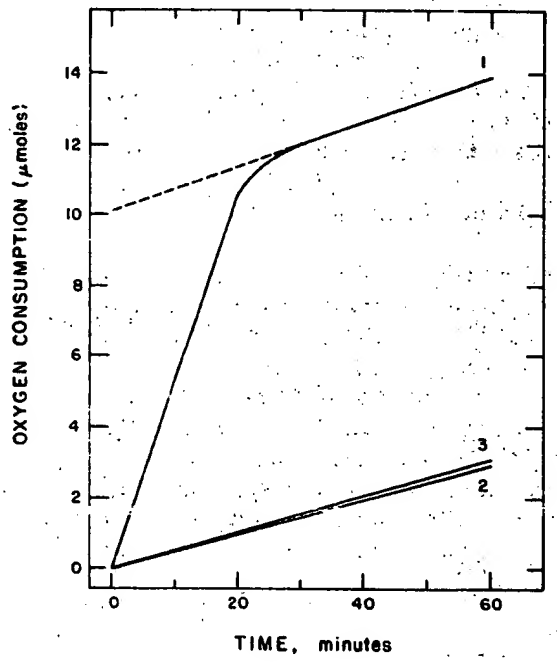

FIG. 6. The consumption of oxygen by 43% AMS in the presence of NADPH and 10 μmoles *p*HPA (1), NADPH and 10 μmoles h mogentisic acid (2), and NADPH only (3). Reaction conditions are the same as described for Table 6.

TABLE 6

The stoichiometry of the conversi n of *p*HPA to h mogentisic acid

Expt.	Alteration to the standard reaction mixture*	Moles oxygen consumed	M les homogentisic acid recovered
A	None	11.5	7.0
	None	10.8	6.7
	10 moles of homogentisic acid instead of *p*HPA	0.2†	8.7
B	None	10	8.5
	None	9.7	7.5
	10 moles of homogentisic acid instead of *p*HPA and minus G-6-P, NADPH	(trace)	7.8
C	2.5 moles NADPH, no G-6-P, NADP	2.6	Not determined
	5.0 moles NADPH, no G-6-P, NADP	4.5	Not determined
	10.0 moles NADPH, no G-6-P, NADP	8.1	Not determined

*The standard reaction mixture contained the following: 100 μmoles bicine buffer, pH 7.0; 10 μmoles $MgSO_4$; 0.025 μmoles FAD; 5 μmoles α,α-dipyridyl; 10 μmoles NADPH; 30 μmoles glucose-6-phosphate (G-6-P); 5 units glucose-6-phosphate dehydrogenase; 10 μmoles *p*HPA; and 5 units of *p*HPA-hydroxylase in a final volume of 3.0 ml. Oxygen consumption was measured in the Warburg apparatus. After the reaction was complete, the reaction mixture was acidified, extracted three times with 20 ml ethylacetate, evaporated to dryness, and dissolved in 5.0 ml water and assayed for homogentisic acid. The extraction procedure gave 98% recovery of homogentisic acid added after acidification to reaction mixtures containing no substrate.

†Total oxygen consumed after 1 h, corrected for endogenous values.

NADH or a NADH-generating system were unsuccessful because of the high consumption of oxygen in the absence of *p*HPA. NADPH, generated from NADP by the action of glucose-6-phosphate dehydrogenase on glucose-6-phosphate, proved to be satisfactory. Sufficient 43% AMS enzyme preparation was used to complete the reaction in 20–30 min, and α,α-dipyridyl was added to inhibit the oxidation of homogentisic acid. The net consumption of oxygen was determined by extrapolation of the oxygen consumption vs. time plot (Fig. 6), and agreed with the value obtained by correcting for endogenous oxygen consumption. The agreement of the two values demonstrated that *p*HPA was not being oxidized beyond homogentisic acid.

In experiments with excess *p*HPA and limiting amounts of NADPH, about 1 mole of oxygen was consumed per mole of NADPH (Table 6). When the supply of NADPH was in excess, 10 μmoles *p*HPA required about 10 μmoles oxygen. Under the conditions of the experiment there was a loss of added homogentisic acid (15–20%) although there was negligible consumption of oxygen. If these losses are applied as corrections to the values found for homogentisic acid produced from *p*HPA, then the oxidation of 1 mole *p*HPA results in the production of 1 mole of homogentisic acid. The enzymatic oxidation f *p*HPA occurred without the pr duction f carbon dioxide. The data obtained satisfy the stoichiometry of the following equation:

$$p\text{-hydroxyphenylacetate} + O_2 + NADPH_2 \rightarrow \text{homogentisic acid} + H_2O + NADP.$$

Discussion

The conversion of *p*HPA to homogentisic acid requires a shift in position of either the hydroxyl group or the acetic acid side chain. The enzymatic reaction described in this paper does not involve dehydroxylation and hydroxylation of the substrate because neither *o*- or *m*-hydroxyphenylacetic acid are intermediates. A consideration of the stoichiometry of the reaction suggests that the shift is accompanied by an oxidation. Since an oxidative shift of the hydroxyl group would result in the production of DHPA whereas the oxidative shift of the acetic acid side chain would produce homogentisic acid, it is concluded that the reaction involves an oxidative shift of the acetic acid side chain.

The metabolism of *p*HPA by an oxidative shift of the side chain to form homogentisic acid has not been previously described. The reaction is analogous to the conversion of *p*-hydroxyphenylpyruvic acid to homogentisic acid by *p*-hydroxyphenylpyruvate hydroxylase (EC. 1.14. 2.2) from mammalian tissues, which has been studied in detail. The conversion of *p*-hydroxy-

phenylpyruvic acid t homogentisic acid by microorganisms does not appear to have been described in the literature. In studies on the microbial catabolism f tyrosine via the conversion to homogentisic acid, it has been generally assumed that *p*-hydroxyphenylpyruvate was involved as an intermediate. The enzyme preparation used in the present studies has low activity with *p*-hydroxyphenylpyruvate, and enzyme fractionation studies indicate that the activities on *p*HPA and *p*-hydroxyphenylpyruvic acid are due to the same enzyme system. Attempts to demonstrate the presence of a *p*-hydroxyphenylpyruvate hydroxylase in the organism after growth on tyrosine have been unsuccessful. Indeed, the organism appears unable to use *p*-hydroxyphenylpyruvate as a substrate or energy source. Earlier studies (16) indicated a requirement for a *p*-hydroxy group in the substrate for the mammalian *p*-hydroxyphenylpyruvate hydroxylase; more recently Taniguchi *et al.* (27) have shown the conversions of phenylpyruvic acid to *o*-hydroxyphenylacetic acid, and of 4-fluorophenylpyruvic acid to 5-fluoro-2-hydroxyphenylacetic acid by a mammalian enzyme which may be identical with *p*-hydroxyphenylpyruvate hydroxylase. Kindl (19) has demonstrated the conversion of phenylpyruvate to *o*-hydroxyphenylacetate in species of higher plants. The mechanism of *p*-hydroxyphenylpyruvate hydroxylase reaction has been investigated with labeled oxygen (29) and discussed by Crandall (5) and more recently by Lindblad *et al.* (21). The reaction appears to be concerted, resulting in the oxidative shift of side chain and the elimination of carbon dioxide.

The mechanism of reaction for the conversion of *p*HPA to homogentisic acid may be similar to that described for *p*-hydroxyphenylpyruvate-1-hydroxylase. However, the reaction described in the present paper appears to have different properties than the mammalian *p*-hydroxyphenylpyruvate hydroxylase. *p*-Hydroxyphenylacetate-1-hydroxylase requires a reduced pyridine nucleotide, Mg^{2+}, and FAD for maximum activity, whereas the mammalian enzyme requires no cofactors other than a non-specific reductant such as ascorbic acid or reduced glutathione plus 2,6-dichlorophenolindophenol for activity. Such non-specific reductants are inactive f r the oxidati n of either *p*HPA or *p*-hydroxyphenylpyruvic acid by the *p*-hydr xyphenylacetate hydroxylase described in this paper.

The *p*-hydroxyphenylacetate-1-hydroxylase has a high specificity for *p*HPA. The enzyme appears to have an absolute requirement for a *p*-hydroxyl group and a high preference for the acetic acid side chain. Thus, *p*-hydroxyphenylpyruvic acid is a relatively poor substrate for the enzyme reaction. Homogentisic acid has been detected as a product of the reaction with *p*-hydroxyphenylpyruvic acid but detailed studies of this reaction have not been carried out.

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